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**10/089696**

INTERNATIONAL APPLICATION NO.

PCT/Jp00/05213

INTERNATIONAL FILING DATE

August 3, 2000

PRIORITY DATE CLAIMED

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TITLE OF INVENTION

METHOD FOR PROMOTING EFFICIENCY OF GENE INTRODUCTION INTO PLANT CELLS

APPLICANT(S) FOR DO/EO/US

HIEI, Yukoh; KASAOA, Keisuke; ISHIDA, Yuji

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau. WO 02/012520
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - ✓ a. ☒ is transmitted herewith.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98, Form PTO-1449(s), and International Search Report (PCT/ISA/210) with 0 cited document(s).
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:
  - ✓ 1.) PCT/IB/308
  - ✓ 2.) Four (4) sheets of Formal Drawings

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PATENT  
0760-0304P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: HIEI, Yukoh et al.  
Int'l. Appl. No.: PCT/JP00/05213  
Appl. No.: New Group:  
Filed: April 3, 2002 Examiner:  
For: METHOD FOR PROMOTING EFFICIENCY OF  
GENE INTRODUCTION INTO PLANT CELLS

PRELIMINARY AMENDMENT

**BOX PATENT APPLICATION**

Assistant Commissioner for Patents  
Washington, DC 20231

April 3, 2002

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

**AMENDMENTS**

**IN THE SPECIFICATION:**

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/JP00/05213 which has an International filing date of August 3, 2000, which designated the United States of America.--

Docket No. 0760-0304P

IN THE CLAIMS:

Please amend the claims as follows:

6. (Amended) The method according to claim 1 or 2, wherein said centrifugation is carried out for 1 second to 4 hours.

8. (Amended) A method for preparing a plant characterized by using the method according to claim 1 or 2.

9. (Amended) Plant cells, plant tissue or plant prepared by the method according to claim 1 or 2.

10. (Amended) The method according to claim 1 or 2, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.

12. (Amended) Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 10.

15. (Amended) The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 13.

18. (Amended) The cells of the plant belonging to family Gramineae, the tissue of the plant belonging to family Gramineae prepared by the method according to claim 16.

Docket No. 0760-0304P

21. (Amended) Rice cells, rice tissue, rice, maize cells, maize tissue or maize prepared by the method according to claim 19.



VERSION WITH MARKINGS TO SHOW CHANGES MADE

The claims have been amended as follows:

6. (Amended) The method according to [any one of claims 1 to 5]claim 1 or 2, wherein said centrifugation is carried out for 1 second to 4 hours.

8. (Amended) A method for preparing a plant characterized by using the method according to [claim 1 to 7]claim 1 or 2.

9. (Amended) Plant cells, plant tissue or plant prepared by the method according to [claim 1 to 8]claim 1 or 2.

10. (Amended) The method according to [any one of claims 1 to 7]claim 1 or 2, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.

12. (Amended) Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 10[ or 11].

15. (Amended) The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 13[ or 14].





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JC13 Rec'd PCT/PTO 03 APR 2002

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## SPECIFICATION

### Method for Promoting Efficiency of Gene Introduction into Plant Cells

#### Technical Field

The present invention relates to a method for promoting efficiency of gene  
5 introduction into plant cells.

#### Background Art

The method for transformation using *Agrobacterium* has a number of  
excellent features including, in general, the high efficiency, the small number of  
copies of the introduced gene, the feature that the gene may be introduced without  
10 fragmenting a specific region called T-DNA, and the feature that the frequency of  
mutation occurred during cultivation is low because transformants may be obtained  
by cultivation for a short period of time. Therefore, the method is widely used as  
the most useful method for transforming various plants.

Although the *Agrobacterium* method is an extremely excellent method for  
5 transforming plants, whether the transformation is successful or not and the  
transformation efficiency largely varies depending on the plant species, genotype and  
the plant tissue used (Potrykus et al. 1998 (Reference (33))). That is, there are  
species with which the transformation has not been successful, and species with  
which the transformation may be attained only with limited varieties. Further, there  
10 are species with which the tissue to be used is limited so that a large amount of  
materials cannot be treated. To prepare a practical variety by genetic recombination,  
it is necessary to prepare a large number of transformed plants and to select the line  
having the desired character therefrom. However, at present, the type of plants with  
which a large number of transformed plants may be prepared for this purpose is  
15 limited. Thus, to develop an improved method by which this problem may be  
overcome is strongly demanded.

Although the method for transformation via *Agrobacterium* differs in the

starting material, composition of the culture medium and the like, it is almost common to the *Agrobacterium* method that the method comprises making a tissue which is a starting material contact a suspension of *Agrobacterium*, selecting transformed cells after co-culturing, and growing transformed plants. The *Agrobacterium* is infected without performing a special treatment except for sterilization treatment which is carried out as required (Rogers et al. 1988 (Reference (34)), Visser 1991 (Reference (38)), McCormick 1991 (Reference (29)), Lindsey et al. 1991 (Reference (28))). Thus, studies for improving transformation system has been carried out mainly on the *Agrobacterium* strain, constitution of the vector, composition of medium, types of selection marker gene and promoter, the type of the tissue used as the material, and the like.

On the other hand, studies for changing the plant tissue before infection of *Agrobacterium* to a physiological state in which the genes are likely to be introduced have been scarcely made. If the physiological state of the tissue can be changed to such a physiological state by a simple treatment, the method is very useful, and it is expected that, in addition to the promotion of the transformation efficiency, transformation may be attained for the species or genotypes with which transformation has been hitherto difficult, that is a prominent effect. Known studies about pretreatment of plant tissue include particle gun treatment (Bidney et al., 1992 (Reference (5))) and ultrasonication treatment (Trick et al., 1997 (Reference (37))). Both of these methods aim at promoting invasion of bacteria into the plant tissue by physically injuring the tissue, so as to increase the number of plant cells infected. However, these methods are nothing more than developments of the leaf disk method (Horsch et al., 1985 (Reference (17))) and not treatments based on novel concepts. The degree of effectiveness and universality of the methods have not been clarified, and they are not used as general methods.

#### Disclosure of the Invention

Accordingly, an object of the present invention is to provide a method for promoting efficiency of gene introduction into plant cells, by which gene introduction can be attained simply with a higher efficiency than the conventional gene introduction by *Agrobacterium* method, without injuring the tissue.

5 The present inventors intensively studied to discover that in the gene introduction method using *Agrobacterium*, the gene introduction efficiency may be significantly promoted by centrifuging the plant cells or plant tissue subjected to the gene introduction, thereby completing the present invention.

That is, the present invention provides a method for promoting efficiency of gene introduction into plant cells by a bacterium belonging to genus *Agrobacterium*, comprising centrifuging said plant cells or plant tissue.

By the present invention, a method for promoting efficiency of gene introduction into plant cells, by which gene introduction can be attained simply with a higher efficiency than the conventional gene introduction by *Agrobacterium* method, without injuring the tissue, has been provided. The method of the present invention may be applied to both monocotyledons and dicotyledons.

#### Brief Description of the Drawings

Fig. 1 is a drawing for showing a method for constructing pTOK233 which is an example of super-binary vectors, that may preferably be employed in the present invention.

Fig. 2 is a gene map of pSB133 which is an example of super-binary vectors, that may preferably be employed in the present invention.

Fig. 3 is a schematic view for showing the intermediate vector system and binary vector system which are major two vector systems of bacteria belonging to genus *Agrobacterium*.

Fig. 4 is a schematic view showing two binary vector systems derived from super virulent strain A281 of *Agrobacterium tumefaciens*.

In the above drawings, the following reference symbols denote the following meanings.

virB: the *virB* gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

5 virC: the *virC* gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

virG: the *virG* gene in the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281

0 BL: left border sequence of T-DNA of bacteria belonging to genus *Agrobacterium*

BR: right border sequence of T-DNA of bacteria belonging to genus *Agrobacterium*

TC: tetracycline resistant gene

SP: spectinomycin resistant gene

5 IG: intron GUS gene

HPT: hygromycin resistant gene

K: restriction enzyme *Kpn* I site

H: restriction enzyme *Hind* III site

Ampr: ampicillin resistant gene

0 BAR: bar gene

Pnos: promoter of nopaline synthetase gene

Tnos: terminator of nopaline synthetase gene

P35S: CaMV 35S promoter

COS, cos: COS site of  $\lambda$  phage

15 ORI, ori: replication origin of ColE1

NPT, NPTII kanamycin resistant gene

Vir: entire *vir* region of Ti plasmid of bacteria belonging to genus

*Agrobacterium*

S Vir: entire *vir* region of Ti plasmid pTiBo542 of super virulent bacteria belonging to genus *Agrobacterium*

s vir\*: fragment containing a part of *vir* region of Ti plasmid pTiBo542

### Best Mode for Carrying out the Invention

The method of the present invention for promoting efficiency of gene introduction into plant cells by a bacterium belonging to genus *Agrobacterium*, comprises centrifuging the plant cells or plant tissue. The plant cells or plant tissue may be contacted with the bacterium belonging to genus *Agrobacterium* under normal gravity after centrifuging the plant cells or tissue, or the plant cells or tissue may be contacted with the bacterium belonging to genus *Agrobacterium* while centrifuging the plant cells or tissue. Preferably, the plant cells or plant tissue are(is) contacted with the bacterium belonging to genus *Agrobacterium* under normal gravity after centrifuging the plant cells or tissue.

The conditions for centrifugation may appropriately be selected depending on the type of the plant used and the like, and may usually be carried out under a centrifugation acceleration of 100G to 250,000G, preferably 500G to 200,000G, more preferably 1000G to 150,000G. The time for centrifugation may appropriately be selected depending on the centrifugal acceleration, type of the plant used and so on, and is usually and preferably not less than one second. There is no upper limit of the centrifugation time, and about 10 minutes may usually be sufficient for attaining the object of the centrifugation. When the centrifugal acceleration is large, the efficiency of introducing genes may be significantly promoted even if the centrifugation time is very short, for example, 1 second or less. On the other hand, when the centrifugal acceleration is small, the efficiency of introducing genes may be significantly promoted by conducting the centrifugation for a long time. In most cases, especially preferred centrifugation conditions are about 500G to 200,000G,

especially 1000G to 150,000G for about 1 second to 2 hours, and the appropriate centrifugation conditions for the particular plant cells or tissue may be easily selected by a routine experiment.

The method of the present invention is characterized by using the plant cells or plant tissue which were(was) centrifuged, or by contacting the plant cells or plant tissue with a bacterium belonging to the genus *Agrobacterium* while conducting centrifugation, and as the method for gene introduction or transformation *per se* using the bacterium belonging to the genus *Agrobacterium*, a well-known method may be applied as it is.

The method for gene introduction or transformation *per se* into plants using a bacterium belonging to the genus *Agrobacterium* is well-known in the art and is widely used.

It is known for a long time that a soil bacterium *Agrobacterium* (*Agrobacterium tumefaciens*) causes crown gall disease in a number of dicotyledons.

In 1970s, it was discovered that Ti plasmid concerns the virulence, and that the T-DNA which is a part of Ti plasmid is incorporated into the plant genome.

Thereafter, it was proved that the T-DNA contains genes participating in synthesis of hormones (cytokinins and auxins) required for induction of tumor, and that the genes are expressed in plants in spite of the fact that the genes are bacterial genes. A

group of genes existing in the virulence region (*vir* region) in the Ti plasmid is required for the excision of T-DNA and its transfer to plants, and the border sequences existing at the both ends of the T-DNA are necessary for the T-DNA to be excised. *Agrobacterium rhizogenes* which is another bacterium belonging to the genus *Agrobacterium* has a similar system on the Ri plasmid (Figs. 3 and 4).

Since T-DNA is incorporated into the plant genome by infection of *Agrobacterium*, it was expected that a desired gene may be incorporated into the plant genome by inserting the desired gene in the T-DNA. However, since Ti

plasmid is as large as not less than 190 kb, it was difficult to insert a gene into the T-DNA by a standard technique of genetic engineering. Thus, a method for introducing a foreign gene into the T-DNA was developed.

First, disarmed strains such as LBA4404 (Hoekema et al., 1983 (Reference (12))), C58C1(pGV3850) (Zambryski et al., 1983 (Reference (40))), and GV3Ti11SE (Fraley et al., 1985 (Reference (9))), that have tumorigenic Ti plasmids from which hormone synthetase genes were eliminated, were prepared (Fig. 3). Two methods employing such a strain, that is, a method by which a desired gene is introduced into the Ti plasmid of *Agrobacterium*, and a method by which a T-DNA having a desired gene is introduced into *Agrobacterium*, were developed. One of these methods is the so called intermediate vector method (Fraley et al., 1985 (Reference (9))); Fraley et al., 1983 (Reference (10))); Zambryski et al., 1983 (Reference (40))), Japanese Laid-open Patent Application (Kokai) No. 59-140885 (EP116718)). In this method, an intermediate vector which is easy to handle by genetic manipulation techniques, in which a desired gene may be inserted, and which can be replicated in *E. coli* is introduced into the T-DNA in the disarmed type Ti plasmid of *Agrobacterium* by triparental mating (Ditta et al., 1980 (Reference (8))). Another method is the so called binary vector method (Fig. 3), which is based on the fact that although the *vir* region is necessary for the T-DNA to be incorporated into plants, it is not necessary that the T-DNA and the *vir* region exist in the same plasmid ((Hoekema et al., 1983). The *vir* region contains *virA*, *virB*, *virC*, *virD*, *virE* and *virG* (Plant Biotechnology Encyclopedia (Enterprise Co., Ltd. (1989))), and the *vir* region is defined as those containing all of *virA*, *virB*, *virC*, *virD*, *virE* and *virG*. Thus, the binary vector is a small plasmid which is replicable in both *Agrobacterium* and *E. coli*, and this plasmid is introduced into *Agrobacterium* having a disarmed type Ti plasmid. The introduction of the binary vector into *Agrobacterium* may be carried out by electroporation method, triparental mating or the like). Binary vector includes

pBIN19 (Bevan, 1984 (Reference (4))), pBI121 (Jefferson, 1987 (Reference (19))), pGA482 (An et al., 1988 (Reference (2))), Japanese Laid-open Patent Application (Kokai) No. 60-70080 (EP120516)), and a number of new binary vectors have been constructed based on these vectors. In the system of Ri plasmid, similar vectors have been constructed and are used for transformation.

*Agrobacterium* A281 (Watson et al., 1975 (Reference (39))) is a super-virulent strain, whose host spectrum is wide and whose efficiency of transformation is higher than other strains (Hood et al., 1987(Reference (13)); Komari, 1989 (Reference (21))). This feature is brought about by a Ti plasmid pTiBo542 contained in A281 (Hood et al., 1984 (Reference (16)); Jin et al., 1987 (Reference (20)); Komari et al., 1986 (Reference (24))).

Two new systems using pTiBo542 has been developed. One system utilizes strains EHA101 (Hood et al., 1986) and EHA105 (Hood et al., 1993) containing a Ti plasmid which is a disarmed type of pTiBo542. By applying these strains to the above-mentioned binary vector system, a system having a high efficiency of transformation was achieved, which is widely used for transformation of various plants. Another system is "super-binary" vector system (Hiei et al., 1994 (Reference (11)); Ishida et al., 1996 (Reference (18)); Komari et al., 1999 (Reference (26)), WO94/00977, WO95/06722) (Fig. 4). Since this system comprises a disarmed type Ti plasmid having the *vir* region (*virA*, *virB*, *virC*, *virD*, *virE* and *virG*) (each of these may also be hereinafter referred to as "*vir* fragment region") and a plasmid having T-DNA, this is a kind of the binary vector system. However, it is different from the binary vector in that a super-binary vector (Komari, 1990a (Reference (22))) in which a *vir* region fragment (preferably a fragment containing at least *virB* or *virG*, more preferably a fragment at least containing *virB* and *virG*) substantially lacking at least one of the fragments of *vir* region is incorporated into the plasmid having the T-DNA, i.e., the binary vector. To introduce a T-DNA region into which a desired



gene has been inserted into an *Agrobacterium* having the super-binary vector, homologous recombination via the triparental mating method may be employed as an easy method (Komari et al., 1996 (Reference (25))). It has been proved that the super-binary vector gives much higher transformation efficiency than the above-described various vector systems for a number of plant species (Hiei et al., 1994 (Reference (11)); Ishida et al., 1996 (Reference (18)); Komari, 1990b (Reference (23)); Li et al., 1996 (Reference (27)); Saito et al., 1992 (Reference (35))).

In the method of the present invention, the host bacterium belonging to the genus *Agrobacterium* is not restricted, and *Agrobacterium tumefaciens* (e.g., the above-described *Agrobacterium tumefaciens* LBA4404 (Hockema et al., 1983 (Reference (12))) and EHA101 (Hood et al., 1986 (Reference (15))) may preferably be employed.

The method of the present invention may be applied to any of the gene introduction systems as long as it is based on the expression of the group of genes in the *vir* region in the bacterium belonging to the genus *Agrobacterium* so as to obtain significant effect. Thus, the method of the present invention may be applied to any of the vector systems such as the above-described intermediate vectors, binary vectors, super-virulent binary vectors and super-binary vectors so as to obtain the advantageous effect of the present invention. The method of the present invention may also be applied to the vector systems obtained by modification of these vectors (e.g., those wherein the entire or a part of the *vir* region of a bacterium belonging to the genus *Agrobacterium* is excised and additionally incorporated into the plasmid, or the entire or a part of the *vir* region of a bacterium belonging to the genus *Agrobacterium* is excised and is introduced into *Agrobacterium* as a part of a new plasmid). Further, needless to say, by the method of the present invention, the efficiency of introduction of the T-DNA region of wild type *Agrobacterium* is promoted so as to promote the infection efficiency.

The desired gene to be introduced into the plant may be inserted into a restriction site in the T-DNA region of the above-described plasmid by a conventional method, and the *Agrobacterium* into which the desired gene was incorporated may be selected based on an appropriate selection marker such as a drug resistant gene against a drug such as kanamycin or paromomycin. In cases where the plasmid is large and has a number of restriction sites, it is not always easy to insert the desired DNA into the T-DNA region by an ordinary subcloning method. In such a case, the desired DNA may be inserted by the triparental mating method utilizing the homologous recombination in the cell of the bacterium belonging to the genus *Agrobacterium*.

Introduction of the plasmid into a bacterium belonging to the genus *Agrobacterium* such as *Agrobacterium tumefaciens* may be carried out by a known method including the above-mentioned triparental mating method, electroporation method, electroinjection method and chemical treatments with PEG or the like.

The gene which is to be introduced into the plant is, in principle, arranged between the left and right border sequences of the T-DNA as in the conventional method. However, since the plasmid is annular, the plasmid may contain only one border sequence. Alternatively, in cases where a plurality of genes are to be arranged at different sites, the plasmid may contain three or more border sequences. Alternatively, arrangement of the desired plasmid in the Ti or Ri plasmid may be performed in the cell of the bacterium belonging to the genus *Agrobacterium*, or the desired gene may be arranged in another plasmid. Further, the desired gene may be arranged in a plurality of types of plasmids.

Introduction of a gene into the plant cells via a bacterium belonging to the genus *Agrobacterium* may be attained by simply making the plant cells or plant tissue contact the bacterium belonging to the genus *Agrobacterium*. For example, a cell suspension of the bacterium belonging to the genus *Agrobacterium* having a

population density of about  $10^6$  to  $10^{11}$  cells/ml is prepared, and the plant cells or the plant tissue are(is) immersed in the suspension for about 3 to 10 minutes, followed by co-culturing the resultant on a solid medium for several days, thereby attaining the introduction of the gene.

5           The cells or the tissue to be subjected to the gene introduction are(is) not restricted at all and may be a leaf, root, stem, fruit or any other portion of the plant. Further, dedifferentiated tissue such as a callus or a non-dedifferentiated tissue such as an embryo may be employed. The type of the plant is not restricted at all, and angiosperms are preferred. As long as the plant is an angiosperm, either  
0           dicotyledon or monocotyledon is preferred.

As will be concretely shown in the following Examples, by the method of the present invention, the efficiency of gene introduction is significantly promoted when compared with the conventional *Agrobacterium* method.

#### Examples

5           The present invention will now be described by way of examples thereof. It should be noted that the present invention is not restricted to the following Examples.

#### Example 1

##### (1) *Agrobacterium* Strains and Plasmids

As the *Agrobacterium* and its vectors, LBA4404(pBI121) (pBI121 is  
0           commercially available from CLONETECH, U.S., (Jefferson RA 1987 (Reference (19))), LBA4404(pIG121Hm) (Hiei, Y. et al., 1994 (Reference (11))), LBA4404(pTOK233) (Hiei et al., 1994 (Reference (11))) and LBA4404(pSB133) (Fig. 2) were used.

Construction of pSB133 was carried out as follows: A DNA fragment  
5           having a size of 6.2 kb obtained by digesting pGA482 (An G et al., 1985 (Reference (3))) with a restriction enzyme *Sal* I was ligated to a DNA fragment with a size of 5.1 kbp obtained by digesting pSB11 (Komari et al., 1996 (Reference (25))) with *Sal* I to

prepare a plasmid. This plasmid was then digested with restriction enzymes *Eco* RI and *Bgl* II to obtain a DNA fragment with a size of 8.6 kb. This DNA fragment was blunted and a *Bgl* II linker (commercially available from TaKaRa) was inserted therein to obtain a plasmid pSB27. The pSB27 was digested with a restriction enzyme *Hind* III, and a DNA fragment with a size of 3.1 kb containing 35S promoter and an intron-GUS gene, which fragment was obtained by digesting pIG221 (Ohta S et al., 1990 (Reference (32))), was inserted therein to obtain pSB33. The pSB33 was introduced into *E. coli* LE392, and then introduced into *Agrobacterium* LBA4404 containing pSB1 (Komari et al., 1996 (Reference (25))) by triparental mating method (Ditta G et al., 1980 (Reference (8))). The pSB133 was obtained by homologous recombination between pSB1 and pSB33 in the cell of *Agrobacterium*. The T-DNA region of pBI121 contains a kanamycin-resistant gene (*nptII*) controlled by the promoter of nopaline synthetase gene (*nos*) and a GUS gene controlled by 35S promoter of cauliflower mosaic virus (CaMV). Each of the T-DNA regions of pIG121Hm and pTOK233 contains an *nptII* gene controlled by *nos* promoter, an *hpt* gene controlled by 35S promoter, and a GUS gene controlled by the 35S promoter, which GUS gene contains introns of the catalase gene of castor bean. The T-DNA region of pSB133 contains an *nptII* gene controlled by *nos* promoter and a GUS gene controlled by 35S promoter of CaMV, which GUS gene contains introns of the catalase gene of castor bean (Fig. 2). The plasmids pSB133 and pTOK233 are super-binary vectors having high abilities of transformation (Komari, T. et al., 1999 (Reference (26))).

As the sample varieties, Koshihikari and Tsukinohikari, which are Japonica rice varieties, were used. Glumes of immature seeds at 8 to 14 days after flowering were removed and the seeds were sterilized with 70% ethanol for several seconds and with 1% aqueous sodium hypochlorite solution containing Tween 20 for 15 minutes.



The co-culturing was carried out for 3 to 13 days, and a portion of the immature embryo was treated with X-Gluc to check the expression of the GUS gene (Hiei et al. 1994) (Reference (11)). That is, immediately after the co-culturing, the tissue was immersed in 0.1M phosphate buffer (pH 6.8) containing 0.1% Triton X-100, and was left to stand at 37°C for 1 hour. After removing *Agrobacterium* with phosphate buffer, phosphate buffer containing 1.0 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc) and 20% methanol was added. After incubating the resultant at 37°C for 24 hours, tissues colored in blue were observed under microscope.

#### (5) Selection of Transformed Cells

After the co-culturing, the immature embryos and calli were transferred to a primary selection medium containing 250 mg/l carbenicillin and 250 mg/l cefotaxime, and further containing 200 mg/l paromomycin or 10 to 30 mg/l hygromycin, and cultured at 30°C under luminous condition for 1 to 2 weeks. As the primary selection medium, 2N6K medium described in Hiei et al. (1994) (Reference (11)) supplemented with D-sorbitol to 30 g/l was used (K medium). Further, a medium (N medium) which was the same as the 2N6 medium (inorganic salts and vitamins of N6 (Chu C. C. 1978 (Reference (7))), 1 g/l casamino acid, 2 mg/l 2,4-D) except that the concentration of  $(\text{NH}_4)_2\text{SO}_4$  was changed to 232 mg/l and that the amino acids of AA medium (Toriyama et al., 1985 (Reference (36))) were supplemented was also used in the test.

The calli formed on the primary selection medium were transferred to a secondary selection medium containing 250 mg/l cefotaxime and 250 mg/l carbenicillin, and further containing 200 mg/l paromomycin or 80 mg/l hygromycin, and cultured at 30°C under luminous condition for 1 to 2 weeks. As the secondary selection medium, a medium which was the same as N6-7 medium described in Hiei et al. (1994) (Reference (11)) except that the concentration of  $(\text{NH}_4)_2\text{SO}_4$  was

changed to 232 mg/l and that the amino acids of AA medium (Toriyama et al., 1985 (Reference (36))) were supplemented was used. To the primary and secondary selection media containing paromomycin, agarose was added to 8 g/l as a solidifier. The rate of emerged resistant calli was investigated after the secondary selection.

#### 5 (6) Regeneration of Transformants

The calli resistant to the selection drugs, obtained from the scutella of immature embryos were plated on N6S3 medium (Hiei et al. 1994 (Reference (11))) for regeneration containing 250 mg/l carbenicillin and 250 mg/l cefotaxime, and further containing 100 mg/l paromomycin or 50 mg/l hygromycin.

#### 0 (7) Checking GUS Expression in Regenerated Plants

Leaves of the regenerated plants resistant to the drugs, obtained by culturing for regeneration at 25°C under luminous condition for 4 to 5 weeks were checked for the expression of GUS gene by treating them with X-Gluc in the same manner as mentioned above (Hiei et al. 1994 (Reference (11))). The regenerated plants were  
5 transplanted to 500-fold diluted aqueous Hyponex solution and cultivated at 25°C under luminous condition for about 2 weeks, followed by transplantation to pots in a green house.

#### (8) Results

##### (i) Discussion about Effects by Centrifugation Treatment

0 Using a micro high-speed centrifuge, large high-speed centrifuge and an ultra high-speed centrifuge, the effect by the centrifugation treatment to the rice immature embryos was tested. As a result, the efficiency of gene introduction was promoted when the acceleration was within the range of 10KG to 100KG (Tables 1, 2, 3 and 6).  
As for the treatment time, advantageous effect was clearly observed with the  
15 treatment for 10 minutes (Tables 4 and 5). The frequency of the transient expression of GUS was not different between the varieties, that is, between Koshihikari and Tsukinohikari. Since not only the effect for promoting the

efficiency of gene introduction, but also the effect for inducing formation of callus was observed, it was suggested that centrifugation treatment is effective for induction and growth of calli and in the culturing of plants including other species.

As shown in Table 6, induction of calli from the immature embryos of Tsukinohikari was not at all observed when the centrifugation was carried out at 250KG for 60 minutes using the ultra high-speed centrifuge. However, induction of calli was observed when the centrifugation was carried out at 110 KG for 60 minutes, and expression of GUS was also observed at high rate. Similarly, as for Koshihikari, induction of calli from the immature embryos of Tsukinohikari was not at all observed when the centrifugation was carried out at 250KG for 60 minutes using the ultra high-speed centrifuge. From these results, the advantageous effect by centrifugation for rice immature embryo is thought to be obtained at an acceleration between 5 KG to 200 KG. Thus, in view of the simplicity of the treatment, when a micro high-speed centrifuge or a large high-speed centrifuge is used, the treatment at 20KG or 40KG is thought to be appropriate. Further, as shown in Tables 9, 10 and 11, it was proved that by the centrifugation treatment at 20KG for 60 minutes, transformation using immature embryo may be attained not only for LBA4404(pSB133) having a super-binary vector known to have a high transformation ability, but also for LBA4404 (pIG121Hm) containing an ordinary binary vector.

## (ii) Discussion about Centrifugation Treatment and Duration of Co-culturing

As shown in Tables 7 and 8, the efficiency of GUS expression observed in the transient assay was higher when the duration of co-culturing was 6 or 13 days than when the duration of co-culturing was 3 days. In another experiment, a high GUS expression was observed when the duration of co-culturing was 9 days. Various immature embryos which underwent different durations of co-culturing are now cultured on a primary selection medium (10 ppm hygromycin, 200 ppm



paromomycin), and there is a tendency that the rate of emerging of drug resistant calli is smaller in the group co-cultured for 9 or 13 days than in the group co-cultured for 3 or 6 days.

(iii) Examination of Efficiency of Transformation by Centrifugation Treatment

At present, the GUS-positive transformants (Tables 4 and 5) prepared as described above are acclimatized, and culturing is continued. For some lines, seeds were collected and fertility was checked. As a result, no differences in morphology and fertility between the centrifuged transformants and the non-treated transformants (Koshihikari and Tsukinohikari) were observed.

Hiei et al. (1994 (Reference (11))) reported that transformation may be attained with a relatively high efficiency using calli of rice. Aldemita RR et al. 1996 (Reference (1))) reported a case of transformation using rice immature embryo. To more effectively and more stably carry out these transformation methods, the above-described centrifugation treatment method is very effective. Especially, although the quality of immature embryo is likely varied depending on the environment of culturing so that it is not easy to always obtain immature embryo suited for transformation, it may be possible to keep high efficiency of transformation by subjecting the immature embryo to the centrifugation treatment. Hiei et al. (1994) (Reference (11)) showed that a super-binary vector having a high transformation ability promotes the efficiency of transformation of rice. According to Aldemita RR et al. 1996 (Reference (1))), transformants were obtained only in the test using LBA4404(pTOK233) containing a super-binary vector. By the centrifugation treatment method according to the present invention, even when an ordinary binary vector is used, a high efficiency of transformation is attained, which is comparable to or even higher than that attained in the transformation using a super-binary vector. Further, by employing both the super-binary vector and the centrifugation treatment method, the efficiency may be even more promoted. Still

further, it is expected that transformants may be obtained by employing the centrifugation treatment method for the varieties with which a transformant has not hitherto been obtained.

Table 1 Various Centrifugation Treatments and Results of GUS Expression after Co-culturing (Sample Strain: LBA4404/pSB133)

Variety	Population Density of Infected Bacterium (cfu/ml)	Not Treated	Centrifugal Acceleration		
			760 G	8,500 G	19,100 G
Koshihikari	$1 \times 10^8$	3/10(+)	6/10(+)	7/10(++)	7/10(+++)
	$1 \times 10^9$	2/10(+)	0/10(-)	4/10(++)	7/10(+++)
Tsukino-hikari	$1 \times 10^8$	4/10(+)	3/10(+)	9/10(+++)	7/10(+++)
	$1 \times 10^9$	1/10(+)	6/10(++)	2/10(+)	7/10(+++)

Time of Centrifugation Treatment: 10 minutes; Duration of Co-culturing: 3 to 5 days;

Number of GUS-positive immature embryos/Number of sample immature embryos

The symbols in parentheses indicate the area of the region in scutella in which GUS was expressed. -: none; +: small; ++: medium; +++: large

Table 2 Rate of Emerging of Paromomycin-resistant Calli from Koshihikari Immature Embryos (Sample Strain: LBA4404/pSB133)

Selection Medium	Population Density of Infected Bacterium (cfu/ml)	Not Treated	Centrifugal Acceleration		
			760 G	8,500 G	19,100 G
N medium	$1 \times 10^8$	4.8%(1/21)	0.0%(0/22)	15.0%(3/20)	31.8%(7/22)
	$1 \times 10^9$	4.3%(1/23)	4.5%(1/22)	16.7%(3/18)	13.3%(2/15)
K medium	$1 \times 10^8$	0.0%(0/21)	0.0%(0/22)	14.3%(3/21)	18.2%(4/22)
	$1 \times 10^9$	0.0%(0/23)	0.0%(0/21)	0.0%(0/19)	0.0%(0/22)

Number of immature embryos from which resistant calli were derived/Number of sample immature embryos, checked after completion of the secondary selection

Time of Centrifugation Treatment: 10 minutes; Duration of Co-culturing: 3 to 5 days

Table 3 Rate of Emerging of Paromomycin-resistant Calli from Tsukinohikari

Immature Embryos (Sample Strain: LBA4404/pSB133)

Selection Medium	Population Density of Infected Bacterium (cfu/ml)	Not Treated	Centrifugal Acceleration		
			760 G	8,500 G	19,100 G
N medium	$1 \times 10^8$	0.0%(0/11)	0.0%(0/11)	30.0%(3/10)	36.4%(4/11)
	$1 \times 10^9$	0.0%(0/11)	9.1%(1/11)	27.3%(3/11)	54.5%(6/11)
K medium	$1 \times 10^8$	0.0%(0/10)	0.0%(0/15)	9.1%(1/11)	9.1%(1/11)
	$1 \times 10^9$	0.0%(0/11)	0.0%(0/11)	0.0%(0/11)	45.5%(5/11)

Number of immature embryos from which resistant calli were derived/Number of sample immature embryos, checked after completion of the secondary selection

- 5 Time of Centrifugation Treatment: 10 minutes; Duration of Co-culturing: 3 to 5 days

Table 4 Time of Centrifugation Treatment and Results of GUS Expression

after Co-culturing

Strains and Plasmids	Not Treated	Time of Centrifugation Treatment		
		10 minutes	30 minutes	60 minutes
LBA4404(pSB133)	9/10(+)	9/10(++)	10/10(++)	10/10(+++)
LBA4404(pTOK233)	9/10(+)	10/10(++)	10/10(++)	10/10(+++)

Centrifugal acceleration: 20,000G; Sample Variety: Koshihikari;

- 0 Number of GUS-positive immature embryos/Number of sample immature embryos  
area of the region in scutella in which GUS was expressed. +: small; ++: medium;  
+++: large



Table 6 Intensity of Centrifugation Treatment and GUS Expression after Co-culturing (variety: Tsukinohikari)

Centrifugation Treatments	Duration of Co-culturing	Number of Immature Embryos			
		Frequency of GUS Expression in Scutella			
		—	±	+	++
Not Treated	3 days	6	4	0	0
	6 days	0	2	6	2
20KG <sup>1)</sup>	3 days	0	0	2	8
	6 days	0	0	2	8
40KG <sup>2)</sup>	3 days	1	0	1	8
	6 days	0	0	0	10
110KG <sup>3)</sup>	3 days	1	0	5	4
	6 days	0	0	2	8
250KG <sup>3)</sup>	3 days	10	0	0	0
	6 days	10	0	0	0

Sample Strain: LBA4404/pIG121Hm; Time of Centrifugation Treatment: 60 minutes

1) micro high-speed centrifuge; 2) large high-speed centrifuge; 3) ultra high-speed centrifuge

Rate of GUS-expressed area in scutella: -: none; ±: <1/8; +: 1/8-1/4; ++: >1/4

Table 7 Centrifugation Treatment, Duration of Co-culturing and GUS Expression after Co-culturing (Variety: Tsukinohikari)

Centrifugation Treatments	Duration of Co-culturing	Number of Immature Embryos			
		Frequency of GUS Expression in Scutella			
		—	±	+	++
Not Treated	3 days	5	4	1	0
	6 days	0	6	2	2
	13 days	0	5	2	3
20KG <sup>1)</sup>	3 days	0	2	5	3
	6 days	0	1	3	6
	13 days	0	1	3	6
40KG <sup>2)</sup>	3 days	0	1	7	2
	6 days	0	0	8	2
	13 days	0	1	5	4

Sample Strain: LBA4404/pIG121Hm; 1) micro high-speed centrifuge; 2) large high-speed centrifuge; Centrifugation was carried out for 60 minutes at the indicated

5 revolution.

Rate of GUS-expressed area in scutella: -: none; ±: <1/8; +: 1/8-1/4; ++: >1/4

Table 8 Centrifugation Treatment, Duration of Co-culturing and GUS Expression after Co-culturing (Variety: Koshihikari)

Centrifugation Treatments	Duration of Co-culturing	Number of Immature Embryos			
		Frequency of GUS Expression in Scutella			
		—	±	+	++
Not Treated	3 days	7	3	0	0
	6 days	3	1	0	0
	13 days	1	6	2	1
20KG <sup>1)</sup>	3 days	0	0	1	9
	6 days	0	0	2	8
	13 days	0	0	1	9
40KG <sup>2)</sup>	3 days	1	0	4	5
	6 days	0	0	0	10
	13 days	0	0	1	9

Sample Strain: LBA4404/pIG121Hm; 1) micro high-speed centrifuge; 2) large high-speed centrifuge; Centrifugation was carried out for 60 minutes at the indicated revolution.

Rate of GUS-expressed area in scutella: -: none; ±: <1/8; +: 1/8-1/4; ++: >1/4

Table 9 Results of Transformation by LBA4404(pBI121) (Variety: Tsukinohikari)

Treatments	Number of Immature Embryos	Number of Acclimatized Plants	Number of GUS-positive Plants	Transformation Efficiency
Not Treated	50	17	12	24.0(%)
Centrifugation Treatment	150	60	54	36.0(%)

Centrifugation Treatment: 20KG-60 minutes; Duration of Co-culturing: 5 days

Table 10 Results of Transformation by LBA4404(pIG121Hm) (Variety: Tsukinohikari)

Treatments	Number of Immature Embryos	Number of Acclimatized Plants	Number of GUS-positive Plants	Transformation Efficiency
Not Treated	40	9	3	7.5(%)
Centrifugation Treatment	47	10	5	10.6(%)

Centrifugation Treatment: 20KG-60 minutes; Duration of Co-culturing: 5 days

5 Table 11 Results of Transformation by LBA4404(pBI121) (Variety: Koshihikari)

Treatments	Number of Immature Embryos	Number of Acclimatized Plants	Number of GUS-positive Plants	Transformation Efficiency
Not Treated	49	4	2	4.1(%)
Centrifugation Treatment	274	35	27	9.9(%)

Centrifugation Treatment: 20KG-60 minutes; Duration of Co-culturing: 5 days

Table 12 Results of Transformation by LBA4404(pSB133) (Variety: Koshihikari)

Treatments	Number of Immature Embryos	Number of Acclimatized Plants	Number of GUS-positive Plants	Transformation Efficiency
Not Treated	63	0	—	0.0(%)
Centrifugation Treatment	281	30	23	8.2(%)

Centrifugation Treatment: 20KG-60 minutes; Duration of Co-culturing: 3 days

### Example 2

Immature embryos of maize with a size of about 1.2 mm (variety: A188, obtained from National Institute of Agrobiological Resources, The Ministry of Agriculture, Forestry and Fisheries) were aseptically collected and washed once with LS-inf liquid medium. To a centrifugal tube containing the immature embryo and



2.0 ml of LS-inf medium containing 100  $\mu$ M acetosyringone, a suspension of *Agrobacterium tumefaciens* LBA4404(pSB131) (Ishida et al. 1996 (Reference (18))) was added to a population density of about  $1 \times 10^9$  cfu/ml, and the resulting mixture was centrifuged at 40,000G, at 4°C for 30 minutes. The control embryo was left to stand in the same cell suspension at room temperature for 30 minutes. After the treatment, the mixture was gently stirred and plated on LS-AS medium such that the surface of hypocotyl contacts the medium. On the other hand, infection to immature embryos after centrifugation treatment was carried out as follows: Embryos aseptically collected were washed once with LS-inf liquid medium and transferred to centrifugation tubes containing the same medium, followed by centrifugation treatment at 20 KG or 40 KG at 4°C for 1 hour. The control sample was left to stand in the liquid medium at room temperature for 1 hour. After the treatment, the liquid medium was removed, and a suspension of LBA4404(pSB131) with a population density of about  $1 \times 10^9$  cfu/ml was added, followed by gently stirring of the mixture. After leaving the mixture to stand at room temperature for 5 minutes, the embryos were plated on LS-AS medium containing 10  $\mu$ M AgNO<sub>3</sub> such that the surface of each hypocotyl contacts the medium. After co-culturing in the dark at 25°C for 3 days, an aliquot of the immature embryos was sampled and the transient expression of the GUS gene was checked by the treatment with X-gluc as in Example 1. The above-described medium and method for culturing were in accordance with Ishida, Y. et al. 1996 (Reference (18)).

The transient expression of the GUS gene in the A188 immature embryos infected with LBA4404(pSB131) is shown in Table 13. Although any embryo showed expression of the GUS gene, a number of the immature embryos subjected to the centrifugation treatment showed expression in larger area than the control immature embryos. The increase in the gene-introduced sites by the centrifugation treatment was observed in both cases wherein the centrifugation treatment was

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# CLAIMS

1. A method for promoting efficiency of gene introduction into plant cells by a bacterium belonging to genus *Agrobacterium*, comprising centrifuging said plant cells or plant tissue.
2. The method according to claim 1, wherein said gene introduction is carried out after centrifuging said plant cells or plant tissue.
3. The method according to claim 1 or 2, wherein the centrifugation is carried out under a centrifugal acceleration of 100G to 250,000G.
4. The method according to claim 3, wherein said centrifugation is carried out under a centrifugal acceleration of 500G to 200,000G.
5. The method according to claim 4, wherein said centrifugation is carried out under a centrifugal acceleration of 1000G to 150,000G.
6. The method according to any one of claims 1 to 5, wherein said centrifugation is carried out for 1 second to 4 hours.
7. The method according to claim 6, wherein said centrifugation is carried out for 5 minutes to 2 hours.
8. A method for preparing a plant characterized by using the method according to claim 1 to 7.
9. Plant cells, plant tissue or plant prepared by the method according to claim 1 to 8.
10. The method according to any one of claims 1 to 7, wherein said plant cells or plant tissue used are(is) originated from an angiosperm.
11. A method for preparing an angiosperm characterized by using the method according to claim 9.
12. Angiosperm cells, angiosperm tissue or angiosperm prepared by the method according to claim 10 or 11.
13. The method according to claim 10, wherein said plant cells or plant tissue

used are(is) originated from a monocotyledon.

14. A method for preparing a monocotyledon characterized by using the method according to claim 11.

15. The monocotyledon cells, monocotyledon tissue or monocotyledon prepared by the method according to claim 13 or 14.

16. The method according to claim 13, wherein said plant cells or plant tissue are(is) originated from a plant belonging to family Gramineae.

17. A method for preparing a plant belonging to family Gramineae characterized by using the method according to claim 13.

18. The cells of the plant belonging to family Gramineae, the tissue of the plant belonging to family Gramineae, or the plant belonging to family Gramineae prepared by the method according to claim 16 or 17.

19. The method according to claim 16, wherein said plant cells or plant tissue are(is) of rice or maize.

20. A method for preparing rice or maize characterized by using the method according to claim 19.

21. Rice cells, rice tissue, rice, maize cells, maize tissue or maize prepared by the method according to claim 19 or 20.



ABSTRACT OF THE DISCLOSURE

A method for gene introduction by which higher efficiency for gene introduction than that by the conventional *Agrobacterium* method may be attained simply and without injuring the tissue is disclosed. According to the method of the present invention, the efficiency of gene introduction into plant cells by a bacterium belonging to genus *Agrobacterium* is promoted by accompanying centrifugation of the plant cells or plant tissue.

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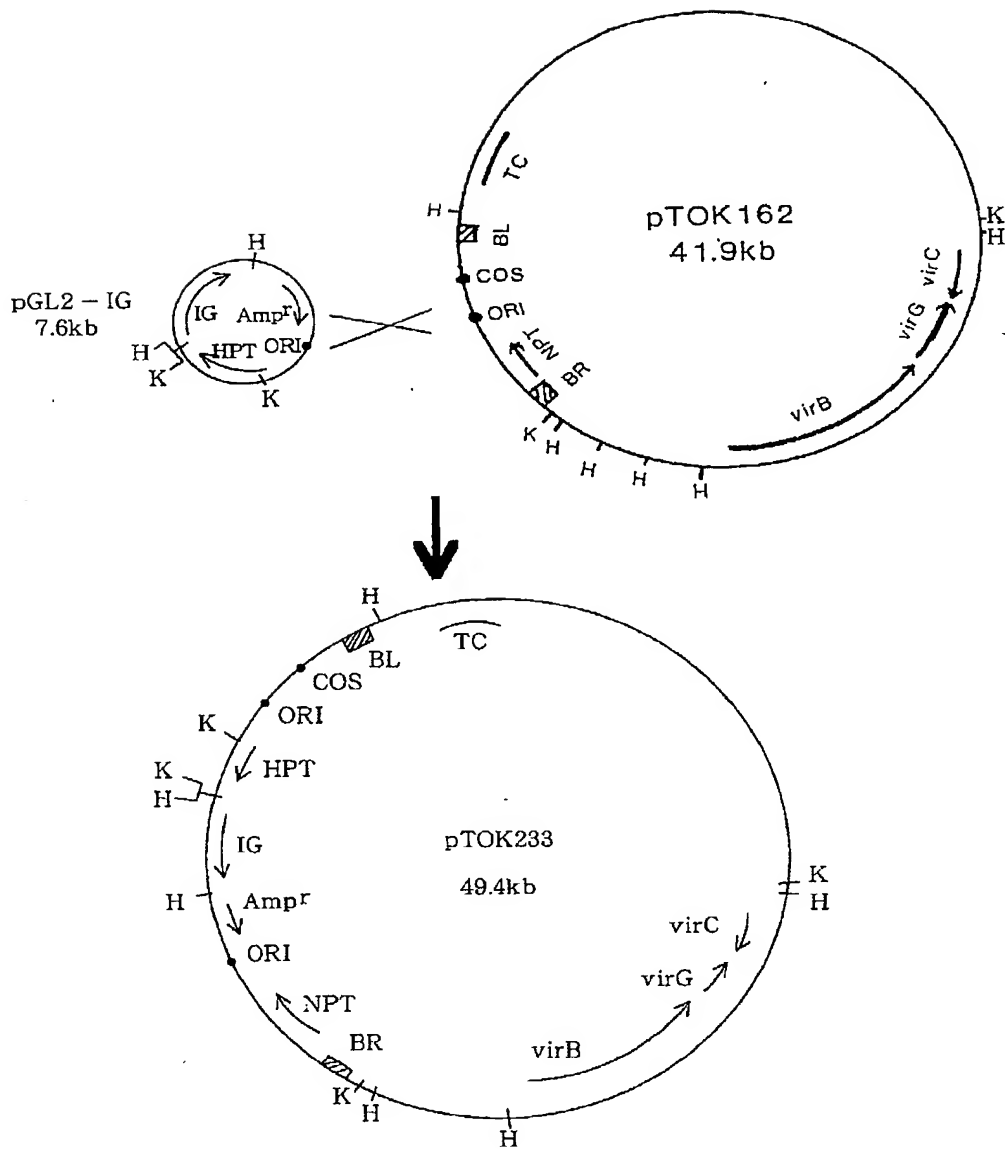


Fig. 1

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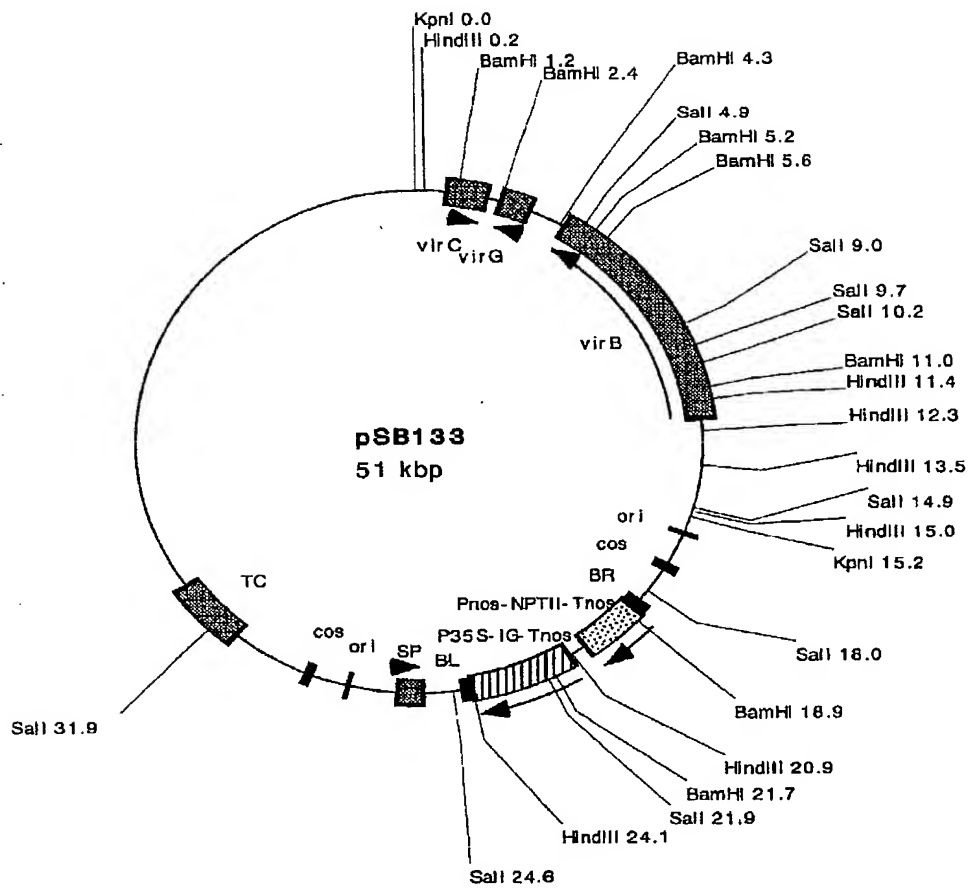


Fig. 2

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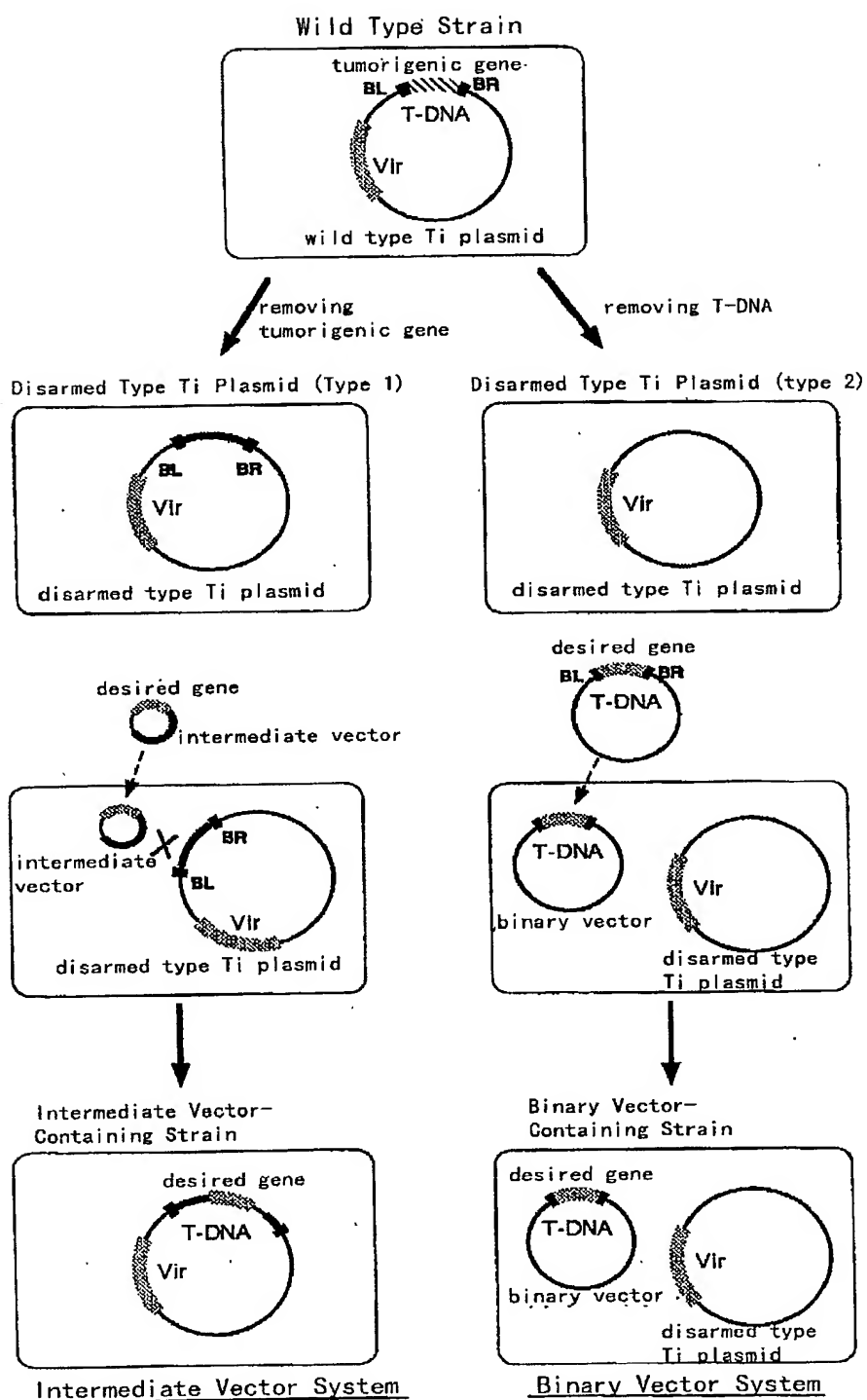


Fig. 3

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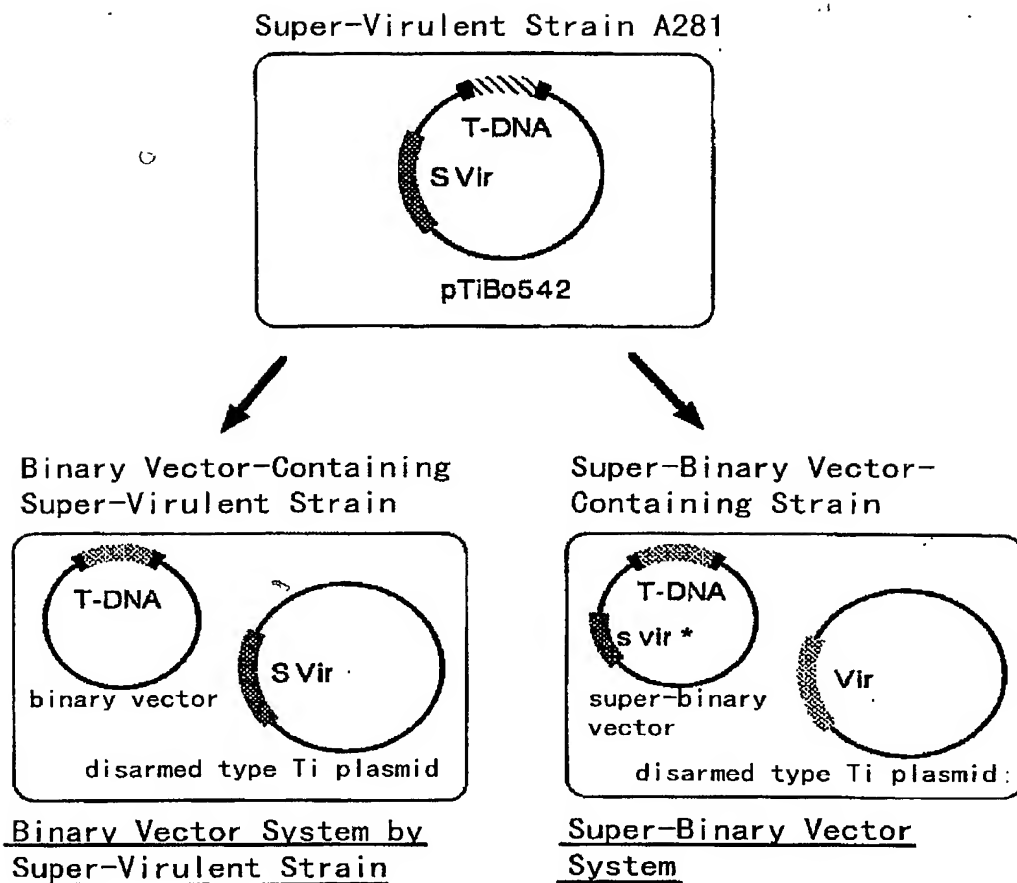


Fig. 4

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Attorney Docket No.

0760-0304P

## COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: "Method for Promoting Efficiency of Gene Introduction into Plant Cells" the specification of which is attached hereto. If not attached hereto,

the specification was filed on \_\_\_\_\_ as  
United States Application Number \_\_\_\_\_ (if applicable) and/or  
and amended on \_\_\_\_\_ as PCT  
the specification was filed on 08/03/00 ✓  
International Application Number PCT/JP00/05213 ✓; and was  
amended under PCT Article 19 on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

<u>158025/99</u>	<u>Japan</u>	<u>06/04/99</u>	No
(Number)	(Country)	(Month/Day/Year Filed)	
_____	_____	_____	
(Number)	(Country)	(Month/Day/Year Filed)	

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below.

_____	_____
(Application Number)	(Filing Date)
_____	_____
(Application Number)	(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Country	Application Number	Date of Filing (Month/Day/Year)
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I hereby claim the benefit under Title 35, United States Code, §120 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

_____	_____	_____
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
_____	_____	_____
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

## Attorney Docket No.

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00	GIVEN NAME/FAMILY NAME <b>Yukoh HIEI</b>	INVENTOR'S SIGNATURE <i>Yukoh Hiei</i>	DATE* Apr. 9, 2002
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	GIVEN NAME/FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
	Residence (City, State & Country)	CITIZENSHIP	
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\*DATE OF SIGNATURE